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TNF-derived peptides for use in treating oedema

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## TNF-DERIVED PEPTIDES FOR USE IN TREATING OEDEMA

### FIELD OF THE INVENTION

5           The present invention is based on the finding that peptides derived from a specific domain of tumor necrosis factor-alpha (TNF- $\alpha$ ) can efficiently be used to treat oedema. More specifically, the present invention relates to the usage of peptides derived from the region of human TNF- $\alpha$  from Ser<sup>100</sup> to Glu<sup>116</sup> to treat pulmonary oedema. For example, the circularized peptide having amino acid sequence CGQRETPEGAEAKPWYC is shown to be very efficient in inducing oedema resorption.

### BACKGROUND OF THE INVENTION

10           Pulmonary transplantation is shown to be successful in the treatment of patients with end-stage pulmonary disease. However, pulmonary oedema or edema (both terms can be used interchangeably) following reperfusion of the transplant is a major clinical problem for which no efficient drug exists at this moment. In addition, recent evidence indicates that the endothelium plays an essential role in regulating the dynamic interaction between pulmonary vasodilatation and vasoconstriction and is a major target during ischemia/reperfusion and acute respiratory distress syndrome (ARDS)-related lung injury. Thus, given that pulmonary edema often results in lung  
20           transplant rejection and that there is a persistent shortage of lungs available for transplantation, there is an urgent need to efficiently prevent or treat pulmonary edema.

25           During ischemia and reperfusion (I/R), a typical induction of inflammatory cytokines like tumor necrosis factor-alpha (TNF) occurs. TNF is a pleiotropic cytokine, mainly produced by activated macrophages, that is synthesized as a transmembrane molecule that can be released by metalloproteinases from the cell surface into the circulation (Gearing *et al.*, 1994). TNF has been shown to bind to at least two types of membrane-bound receptors, TNF receptor 1 (55 kD) and TNF receptor 2 (75 kD), that are expressed on most somatic cells, with the exception of erythrocytes and unstimulated T lymphocytes. TNF can be considered as a two-edged sword: indeed, when overproduced, TNF has been shown to be implicated in the pathology of various infectious diseases,  
30           such as LPS-induced sepsis (Beutler *et al.*, 1985), cerebral malaria (Grau *et al.*, 1987), as well as treatment-associated mortality in African trypanosomiasis (Lucas *et al.*, 1993). In contrast, TNF was shown to be one of the most efficient protective agents against cecal ligation and puncture-induced

septic peritonitis in mice and rats (Echtenacher *et al.*, 1990; Alexander *et al.*, 1991; Lucas *et al.*, 1997) and to be implicated in host defense during pneumococcal pneumonia in mice (van der Poll *et al.*, 1997). Moreover, mice deficient in TNF receptor 1 were shown to be significantly more sensitive to *Listeria monocytogenes* (Rothe *et al.*, 1993; Pfeffer *et al.*, 1993) and *Mycobacterium tuberculosis* infection (Flynn *et al.*, 1995) as well as against fungal (Steinshamn *et al.*, 1996) and *Toxoplasma* infections (Deckert-Schluter *et al.*, 1998). Therefore, it becomes clear that apart from its detrimental effects during overproduction or during prolonged chronic secretion, TNF is also one of the most potent protective agents against infections by various pathogens.

Apart from exerting a plethora of effects mediated by the activation of its two types of receptors (TNF receptor 1, 55kD, and TNF receptor 2, 75 kD), TNF can also mediate receptor-independent activities. The tip domain of TNF is located on the top of its bell-shaped structure and is spatially distinct from its receptor binding sites, that are localized at the base of the trimeric molecule (Lucas *et al.*, 1994). This domain has lectin-like affinity for specific oligosaccharides, such as trimannose and diacetylchitobiose. Both TNF and the tip peptide of TNF are capable of mediating a trypanolytic activity by interfering with the lysosomal integrity of the trypanosome, a pH-dependent effect probably involving the insertion of TNF into the lysosomal membrane (Magez *et al.*, 1997). Moreover, mutants of the tip peptide in which three critical amino acids (T(105); E(107); E(110)) were replaced by A, were completely unable to mediate this activity (Lucas *et al.*, 1994). A mouse TNF (mTNF) triple mutant, T105A-E107A-E110A (referred to hereafter as triple mTNF), lacks the trypanolytic and lectin-like affinity to oligosaccharides as compared to wild type TNF. The triple mTNF has significantly reduced systemic toxicity as compared to wild-type mTNF *in vivo*, but retains its peritonitis-protective effect in a murine model (Lucas *et al.*, 1997).

Another receptor-independent activity of TNF is its membrane-inserting and sodium channel forming capacity (Baldwin *et al.* 1996). Indeed, others have shown that TNF forms a Na<sup>+</sup>-channel in an artificial lipid bilayer model, an activity that is pH-dependent, probably because it requires the «cracking» of the trimer, thus exposing hydrophobic residues to the membrane (Kagan *et al.*, 1992).

Recent observations have indicated that instillation of anti-TNF-neutralizing antibody into the lungs of rats 5 min before bacterial infection inhibits the increase in alveolar liquid clearance, which is known to be driven by a change in intracellular sodium content in the alveolar epithelial cells. Moreover, instillation of TNF in normal rats increases alveolar liquid clearance by 43% over 1 hour (Rezaiguia *et al.*, 1997). Although the latter findings indicate that TNF might be used to induce alveolar liquid clearance, wild type TNF cannot be used therapeutically due to its high systemic

toxicity. The present invention relates to the usage of TNF-derived peptides which can, to our surprise, efficiently be used to induce edema resorption and which have, compared to wild type TNF, lost systemic toxicity.

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### AIMS OF THE INVENTION

It is clear that there is an urgent need to efficiently prevent or treat pulmonary edema. Although some data demonstrate that TNF might be involved in oedema resorption, it is clear that this pleiotropic and potentially toxic molecule can not be used to treat oedema.

10

In this respect, the present invention aims at providing a non-toxic molecule with the same oedema resorption-inducing capacity as TNF. More specifically, the present invention aims at providing non-toxic peptides derived from TNF which can be used to prevent or treat oedema. Moreover, the present invention aims at providing a pharmaceutical composition comprising TNF-derived peptides which induce oedema resorption. In essence, the present invention aims at providing a new medical use of the TNF-derived, trypanocidal peptides as described by Lucas *et al.*

15

(1994) and variants thereof.

All the aims of the present invention are considered to have been met by the embodiments as set out below.

20

### BRIEF DESCRIPTION OF DRAWINGS

**Figure 1** shows a significantly increased inward and outward ion current when murine microvascular endothelial cells were pretreated with the murine, circular TNF-tip peptide (sequence = CGPKDTPEGAELKPWYC) at a concentration of 100 µg/ml at pH = 6.

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mV = millivolt; pA = picoampere.

**Figure 2** shows no increase in ion current when murine microvascular endothelial cells were pretreated with control medium alone; mV = millivolt; pA = picoampere.

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### DETAILED DESCRIPTION OF THE INVENTION

The invention described herein draws on previously published work and pending patent

applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All these publications and applications, cited previously or below are hereby incorporated by reference.

The present invention relates to the use of a peptide comprising a chain of 7 to 17, preferably a chain of 11 to 16, more preferably a chain of 13 to 15 and most preferably a chain of 14 contiguous amino acids derived from the region of human TNF- $\alpha$  from Ser<sup>100</sup> to Glu<sup>116</sup> or from the region of mouse TNF- $\alpha$  from Ser<sup>99</sup> to Glu<sup>115</sup> for the manufacture of a medicament for treating oedema. More specifically the present invention relates to the use of a peptide as described above wherein said chain of 14 contiguous amino acids are chosen from the group consisting of the contiguous amino acid sequences QRETPEGAEAKPWY and PKDTPEGAEELKPWY as described by Lucas *et al.* (1994). The latter sequences are given in the well-known one-letter code for amino acids (the three-letter code is sometimes used further).

The term "peptide" refers to a polymer of amino acids (aa) derived from the trypanolytic TNF domain having lectin-like affinity as described by Lucas *et al.* (1994). Moreover, the latter term relates to a polymer of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or more contiguous amino acids derived from the region of human TNF- $\alpha$  from Ser<sup>100</sup> to Glu<sup>116</sup> or from the region of mouse TNF- $\alpha$  from Ser<sup>99</sup> to Glu<sup>115</sup>. The latter TNF regions refer to the regions shown in Fig. 5, p. 172 of Pennica and Goeddel in Webb and Goeddel, eds. (1987). The term "peptide" may also refer to any peptide comprising the hexamer TPEGAE of the latter TNF regions or any peptide comprising the aa T, E and E of the latter hexamer. It should be clear that the present invention relates to any peptide derived from the latter TNF regions and does not exclude post-translational modifications of the peptides such as glycosylation, acetylation, phosphorylation, modifications with fatty acids and the like. Included within the present invention are, for example, peptides containing one or more analogues of an aa (including unnatural aa's), peptides with substituted linkages, mutated versions or natural sequence variations of the peptides, peptides containing disulfide bounds between cysteine residues, as well as other modifications known in the art. The peptides of the present invention can also be defined functionally, that is, the present invention relates to any peptide which can be used to treat oedema or which can be used for the manufacture of a medicament for treating oedema. In essence, the present invention relates to any molecule, obtained by any method in the art, with the same or very similar characteristics as the trypanolytic peptides defined by Lucas *et al.* (1994).

The peptides of the present invention can be prepared by any method known in the art such as



classical chemical synthesis, as described by Houbenweyl (1974) and Atherton & Shepard (1989), or by means of recombinant DNA techniques as described by Maniatis et al. (1982) and, more specifically, by Lucas et al. (1994).

5 The term oedema (or edema) relates to any abnormal excess accumulation of (serous) fluid in connective tissue or in a serous cavity. In particular, the latter term relates to pulmonary oedema (see also *Examples* section).

Furthermore, the present invention concerns the use of a peptide as described above wherein said peptide is circularized. More specifically, the present invention relates to the use of a peptide as described above, wherein said peptide is circularized by replacing the NH<sub>2</sub>- and COOH-terminal  
10 amino acids by cysteine so that a disulfide bridge is formed between the latter cysteines. In this regard, the present invention concerns the use of a peptide as described above wherein said circularized peptides are chosen from the group consisting of the circularized peptides CGQRETPEGAEAKPWYC and CGPKDTPEGAEELKPWYC as described by Lucas *et al.* (1994).

15 The present invention finally relates to a pharmaceutical composition for treating oedema comprising a peptide as described above. The terms "a pharmaceutical composition for treating oedema" relates to any composition comprising a peptide as defined above which prevents, ameliorates or cures oedema, in particular pulmonary oedema. More specifically, the terms "a pharmaceutical composition for treating oedema" or "a drug or medicament for treating oedema" (both terms can be used interchangeably) relate to a composition comprising a peptide as described  
20 above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat oedema. Suitable carriers or excipients known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the  
25 individual receiving the composition such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers. The "medicament" may be administered by any suitable method within the knowledge of the skilled man. The preferred route of administration is parenterally. In parenteral administration, the medicament of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in  
30 association with the pharmaceutically acceptable excipients as defined above. However, the dosage and mode of administration will depend on the individual. Generally, the medicament is administered so that the peptide of the present invention is given at a dose between 1 µg/kg and 10

mg/kg, more preferably between 10 µg/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous infusion may also be used. If so, the medicament may be infused at a dose between 5 and 20 µg/kg/minute, more preferably between 7 and 15 µg/kg/minute.

5 The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and can not be construed as to restrict the invention in any way.

### 10 EXAMPLES

#### Example 1: TNF peptides induce an ion current in living cells in vitro

Before we started measuring voltage-dependent current with whole cell patch clamp, we characterized CBA mice wild type lung microvascular endothelial cells (MVEC; Lucas *et al*;  
15 submitted for publication) for TNF-induced ELAM-1 expression, TNF-mediated ICAM-1 upregulation and PECAM-1 (CD31) positivity. TNF mediated upregulation of ICAM-1 and induced ELAM-1 expression in CBA mice lung wild type MVEC. These cells were also positive for PECAM (CD31).

#### 20 *A) pH-dependence*

Doing whole cell patch clamp experiments (Bolton *et al.*, 1986), we observed that, following a 30 min pretreatment of the CBA lung MVEC with wt mTNF, we didn't observe an increased voltage-dependent current when the pretreatment was done in NES buffer at pH 7.3. In contrast, a pretreatment for 30 min in NES buffer at pH 6.0 with wt mTNF, at doses of 100 ng/ml and 10  
25 ng/ml, followed by a replacement of the buffer with NES pH 7.3, resulted in a significantly increased outward ion current, as compared to cells pretreated with the control NES buffer at the same pH.

#### *B) Different types of pretreatment:*

30 When lung MVEC were pretreated with TNF for 30 min in NES buffer at pH 6, we observed an increase in outward current. On the other hand, if at this pH TNF is presented to the cells by means of perfusion (added in the perfusion system buffer), no changes in voltage-dependent current

could be detected.

**C) Pretreatment of lung MVEC with wt TNF versus triple mTNF:**

When comparing the pretreatment of lung MVEC with wt mTNF (100 ng/ml) *versus* triple mTNF at the same dose, we observed that the triple mTNF had lost the ion current increasing activity found with wt mTNF. Even a hundredfold increased dose of triple mTNF (1 µg/ml) over wt mTNF (10 ng/ml) caused no significant upregulation of ion current as compared to the control cells.

**D) The ion channel activity of TNF is TNF receptor-independent**

In order to investigate whether the ion channel effect of wt mTNF in mammalian cells is TNF receptor-independent, we used macrophages and lung microvascular endothelial cells isolated from C57BL6 mice deficient in both TNF receptors. As such, we found that both in resident peritoneal macrophages and in lung MVEC isolated from these mice, pretreatment for 30 min with wt mTNF at pH 6 had a similar increasing effect on voltage-dependent current as seen with cells carrying both TNF receptors. Thus, the observed effect of wt TNF is TNF receptor-independent.

**E) Effect of long and short tip peptide on ion current**

Pretreatment of the microvascular endothelial cells with the long, circular tip peptide (17 AA = CGPKDTPEGAELKPWYC, see above) at pH = 6, resulted in a significantly increased inward and outward ion current when added at a concentration of 1 mg/ml or 100 µg/ml (FIG 1) as compared to control cells pretreated with control medium (FIG 2).

**Example 2: Evaluation of TNF peptide in a model of Rat Lung Isotransplantation**

The present study aims at evaluating the potential anti-edematous- and protective effect of a murine TNF peptide on the pulmonary endothelial- and alveolar barrier tissue integrity in a model of lung ischemia/reperfusion injury.

Male Fischer (F344) rats weighing 200 to 250 g undergo orthopic single left lung transplantation after 20 hours cold ischemia using a cuff technique for the vessel anastomoses (Mizuta *et al*, 1989) and a conventional running suture for the bronchial anastomosis. This model is well established in our facility.

Animals are anesthetized by intraperitoneal administration of pentobarbital (50mg/kg) and heparinized (500 I.U./kg). A tracheotomy is performed and the animals are ventilated through a cannula with 100 % O<sub>2</sub> by a Harvard rodent ventilator (Harvard Apparatus, South Natick, Massachusetts) at a tidal volume of 10 ml/kg. After cutting the inferior vena cava and left appendix  
5 of the heart, a small silicon hose is inserted into the main pulmonary artery. Both lungs are flushed with 20 cc of LPD solution (Perfadex, Kabi Pharmacia, Sweden) at a pressure of 20 cm H<sub>2</sub>O. The trachea is then tied in end-inspiration. After removal of the heart-lung block, 14 gage cuffs are placed around the pulmonary artery and vein, and the vessels are inverted and tied onto the cuff. The lung is stored in LPD solution at 4°C until implantation.

10 The recipient is anesthetized by breathing Halothane in a glass chamber, intubated, and anesthesia is maintained with Halothane 2 %. A left lateral thoracotomy is performed in the 4th intercostal space. The left hilum is dissected. After clamping the pulmonary artery and vein with removable microclips, the pulmonary vein is opened, flushed with heparinized saline solution, and the cuff is inserted and fixed with 6-0 Silk. In the same way, the pulmonary artery is anastomosed.  
15 The native left lung is removed and the bronchial anastomosis performed with a running over-and-over suture with 9-0 Monosor® (Auto Suture, Switzerland). The lung is inflated and then reperfused. A chest tube is inserted and the thoracotomy closed. The chest tube is removed after restoration of sufficient spontaneous breathing. Typically, the animals tolerate the procedure well and start eating about one hour after extubation.

20 Each study group (see further, I to IV), with each assessment (see further, a to c), consists of six rats. Group I receives the active, murine TNF tip peptide (17 amino acids, circularized), group II the mutant peptide (17 amino acids, circularized; T104, E106 and E109 are replaced by A), group III wild type murine TNF and group IV serves as control (i.e. receives a non-relevant peptide/protein?). The following assessments are made:

25 a) function of the transplanted lung: arterial blood gas analysis as described in Locke *et al.* (1991), Shiraishi *et al.* (1996) and Chien *et al.* (1997) is performed 24 hours after implantation and following occlusion of the right native lung with micro vessel clips for 5 min.

b) measurement of edema and inflammatory remodeling parameter: albumin leakage is assessed 24  
30 hours after implantation by means of injecting 1 ml intraperitoneally per rat of a 8% human serum albumin solution 1 hour before sacrifice of the animals. Subsequently, an ELISA assay specific for human serum albumin (which has been developed in our laboratory using standard procedures) is performed on the bronchoalveolar lavage fluids of the treated rats.

c) measurement of MPO activity, TBARS, protein content and biochemical parameters: 24 hours after implantation a sternotomy is carried out. About 2-3 ml blood are collected by puncture of the thoracic aorta. The lungs are flushed with 20 ml saline and the transplanted lung is frozen with liquid nitrogen. Myeloperoxidase (MPO) activity is measured according to Chapelier *et al.* (1995), Bacha *et al.* (1997) and Fujino *et al.* (1997). Thiobarbituric acid reaction (TBARS) is performed as previously described by Ohkawa *et al.* (1979), Haniuda *et al.* (1995) and Nagahiro *et al.* (1997). Protein content in bronchoalveolar lavage fluid is measured as previously described by Steinberg *et al.* (1994) and Hamacher *et al.* (1998). As biochemical parameters, (pro-) metalloproteinase (MMP)-9 and (pro) MMP-2 are measured semiquantitatively from bronchoalveolar lavage with gelatin zymography performed under non-reducing conditions on a gel which is then 'incubated' for digestion as has been for example described by Fukuda *et al.* (1998).

The results of this study indicate that the TNF tip peptide has an anti-edematous- and protective effect on the pulmonary endothelial- and alveolar barrier tissue integrity in a model of lung ischemia/reperfusion injury in rats.

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CLAIMS

1. Use of a peptide comprising a chain of 7 to 17 contiguous amino acids derived from the region of human TNF- $\alpha$  from Ser<sup>100</sup> to Glu<sup>116</sup> or from the region of mouse TNF- $\alpha$  from Ser<sup>99</sup> to Glu<sup>115</sup> for the manufacture of a medicament for treating oedema.
2. Use of a peptide according to claim 1, wherein said peptide comprises a chain of 11 to 16 contiguous amino acids.
3. Use of a peptide according to claim 1, wherein said peptide comprises a chain of 13 to 15 contiguous amino acids.
4. Use of a peptide according to claim 1, wherein said peptide comprises a chain of 14 contiguous amino acids.
5. Use of a peptide according to claim 4, wherein said chain of 14 contiguous amino acids are chosen from the group consisting of the contiguous amino acid sequences QRETPEGAEAKPWY and PKDTPEGAEELKPWY.
6. Use of a peptide according to any of claims 1 to 5, wherein said peptide is circularized.
7. Use of a peptide according to claim 6, wherein said peptide is circularized by replacing the NH<sub>2</sub>- and COOH-terminal amino acids by cysteine so that a disulfide bridge is formed between the latter cysteines.
8. Use of a peptide according to claim 7, wherein said circularized peptides are chosen from the group consisting of the circularized peptides CGQRETPEGAEAKPWYC and CGPKDTPEGAEELKPWYC.
9. Use of a peptide according to any of claims 1 to 8, wherein said oedema is pulmonary oedema.
10. A pharmaceutical composition for treating oedema comprising a peptide according to any of claims 1 to 9.

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# ABSTRACT

5 The present invention relates to the finding that peptides derived from a specific domain of tumor necrosis factor-alpha (TNF- $\alpha$ ) can efficiently be used to treat oedema. More specifically, the present invention relates to the usage of peptides derived from the region of human TNF- $\alpha$  from Ser<sup>100</sup> to Glu<sup>116</sup> to treat pulmonary oedema. Moreover, the present invention concerns a circularized peptide having amino acid sequence CGQRETPEGAEAKPWYC which is very efficient in inducing oedema resorption.

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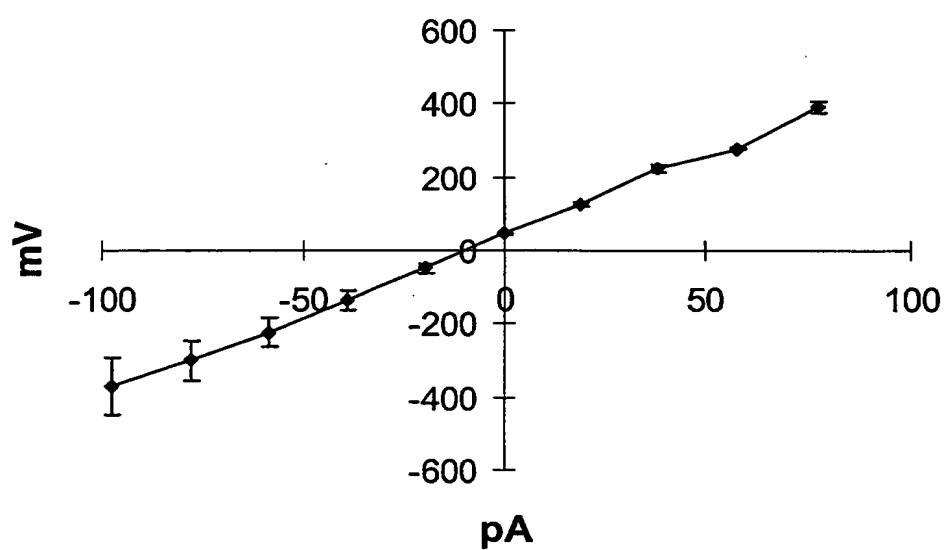


FIG. 1

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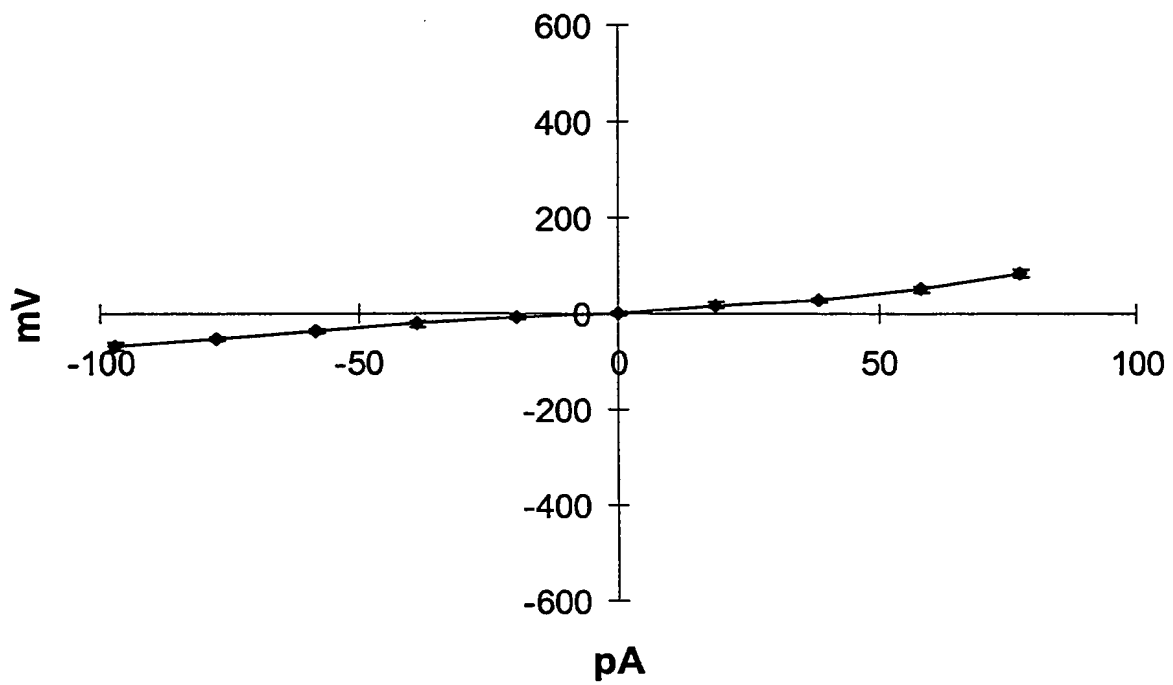


FIG. 2